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Docking of the Periplasmic FecB Binding Protein to the FecCD Transmembrane Proteins in the Ferric Citrate Transport System of *Escherichia coli*[▽]

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Citrate-mediated iron transport across the cytoplasmic membrane is catalyzed by an ABC transporter that consists of the periplasmic binding protein FecB, the transmembrane proteins FecC and FecD, and the ATPase FecE. Salt bridges between glutamate residues of the binding protein and arginine residues of the transmembrane proteins are predicted to mediate the positioning of the substrate-loaded binding protein on the transmembrane protein, based on the crystal structures of the ABC transporter for vitamin B_{12}, consisting of the BtuF binding protein and the BtuCD transmembrane proteins (E. L. Borths et al., Proc. Natl. Acad. Sci. USA 99:16642–16647, 2002). Here, we examined the role of the residues predicted to be involved in salt-bridge formation between FecB and FecCD by substituting these residues with alanine, cysteine, arginine, and glutamate and by analyzing the citrate-mediated iron transport of the mutants. Replacement of E93 in FecB with alanine [FecB(E93A)], cysteine, or arginine nearly abolished citrate-mediated iron transport. Mutation FecB(E222R) nearly eliminated transport, and FecB(E222A) and FecB(E222C) strongly reduced transport. FecD(R54C) and FecD(R51E) abolished transport, whereas other R-to-C mutations in putative interaction sites between FecCD and FecB substantially reduced transport. The introduced cysteine residues in FecB and FecCD also served to examine the formation of disulfide bridges in place of salt bridges between the binding protein and the transmembrane proteins. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis results suggest cross-linking of FecB(E93C) to FecD(R54C) and FecB(E222C) to FecC(R60C). The data are consistent with the proposal that FecB(E93) is contained in the region that binds to FecD and FecB(E222) in the region that binds to FecC.

Fe^{3+}-siderophores, heme, and vitamin B_{12} are translocated across the outer membrane of *Escherichia coli* by proton-motive-force-coupled transporters and are translocated across the cytoplasmic membrane by ATP-dependent ABC transporters. Little is known about the intermediate step involving the periplasmic binding proteins, which interact with transmembrane proteins in the cytoplasmic membrane and donate their substrates to the transmembrane transporters.

The Fe^{3+}-citrate transport system of *E. coli* K-12 consists of the FecA protein in the outer membrane, the FecB binding protein in the periplasm, the FecCD proteins in the cytoplasmic membrane, and the FecE ATPase associated with the cytoplasmic membrane. Fe^{3+} is transported into the periplasm as a diferric dicitrate complex and across the cytoplasmic membrane probably as Fe^{3+}. No cotransport of radiolabeled [$^{55}Fe^{3+}$] with radiolabeled [^{14}C]citrate was observed (3). The *fecABCDE* transport genes are organized in an operon regulated by the Fur repressor and ferric citrate. Binding of diferric dicitrate to the FecA protein (10, 25) elicits a signal that is transduced by FecA across the outer membrane and by FecR across the cytoplasmic membrane into the cytoplasm. FecA interacts with its periplasmic N domain (7, 11) and with the periplasmic C domain of FecR. The FecI sigma factor is thereby activated and directs RNA polymerase to the promoter upstream of *fecA*. Cells respond to iron limitation economically by synthesizing the FecIR regulatory proteins upon iron limitation and by synthesizing the transport system only when the substrate is in the medium. The novel regulation of ferric citrate transcription control has been studied in some detail, but few investigations have been concerned with the transport mechanism (summarized in references 3, 4, 5, and 6). *E. coli* K-12 acquired the Fec transport system via horizontal gene transfer (16).

Here we used the Fe^{3+}-citrate transport system to study the open question of how binding proteins dock to transmembrane proteins of bacterial ABC transporters. The structural basis for understanding the mode of action of the ABC transporter FecBCDE is provided by the first crystal structure of an ABC transporter published, that of the BtuCD transporter for vitamin B_{12} (15). The crystal structure of the vitamin B_{12} binding protein of *E. coli* was determined (2) separately from that of the BtuCD transmembrane transporter. The two structures can be assembled such that one glutamate residue of BtuF forms salt bridges with one of three possible arginine residues of one of the two BtuC monomers, and another glutamate residue of BtuF forms salt bridges with one of these arginine residues in the other monomer (Fig. 1). The combined structure predicts how BtuF interacts with BtuC when it donates vitamin B_{12} to the BtuC dimer. In many respects, the vitamin B_{12} transport system is very similar to the Fe^{3+}-siderophore

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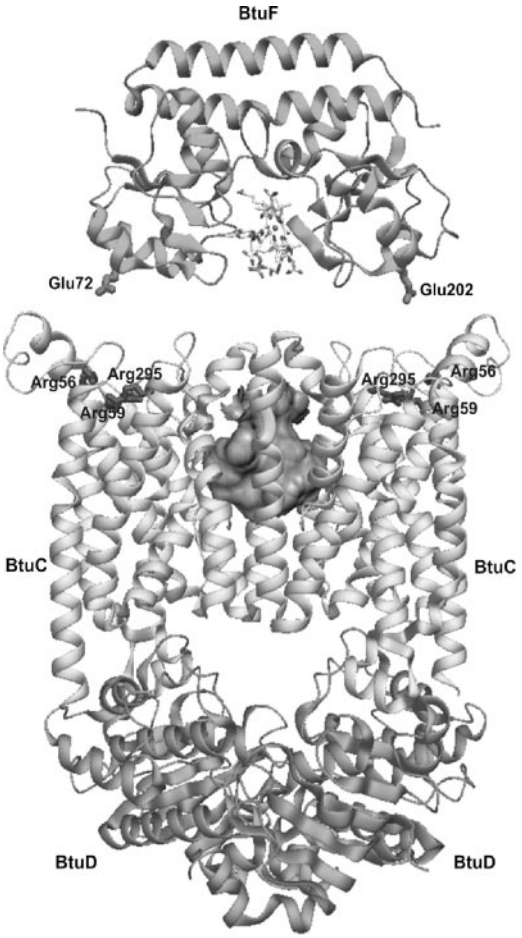

FIG. 1. Crystal structures of the BtuF periplasmic binding protein of vitamin B_{12} transport (2), the BtuC transmembrane dimer, and the BtuD ATPase (15), as assembled by Borths et al. (2), modified by labeling the amino acids proposed to form salt bridges between BtuF and BtuC.

transport systems. For example, the predicted interacting amino acids are conserved in most Fe^{3+}-siderophore transporters (Fig. 2). Here, we analyzed whether single mutations in the potential interaction sites between the binding protein and the transmembrane proteins of the Fe^{3+}-citrate transport system affect transport.

MATERIALS AND METHODS

Strains and growth conditions. *E. coli* K-12 was grown in TY liquid medium, which contains (per liter) 10 g of Bacto tryptone (Difco Laboratories), 5 g of yeast extract, and 5 g of sodium chloride (pH 7)/liter, or on TY nutrient agar plates. Transformants were selected by adding ampicillin (40 μg/ml) or chloramphenicol (20 μg/ml).

Construction of *fecB*, *fecC*, and *fecD* mutants. The *fecIR* regulatory genes and *fecABCDE* transport genes were cloned in plasmid pSV662, a pHSG576 derivative (12). To remove the SalI site of pHSG576, vector DNA was cleaved with SalI, and the ends were filled in with Klenow enzyme, resulting in pHSG76S. The *fecIRABCDE* fragment was excised from pSV662 and cloned into pHSG76S, resulting in pFO76S.

The *fecB*, *fecC*, and *fecD* genes were mutagenized by PCR using a QuikChange site-directed mutagenesis kit with *Pfu* Turbo polymerase (Stratagene). The primers were designed such that in most cases two nucleotides were changed in codons to reduce spontaneous mutations back to wild-type. The plasmids used are available on request. In addition, freshly transformed mutant cells were used in all experiments to avoid selection for suppressor mutations. For *fecB* mutagenesis, plasmid pAS87 was used; the plasmid contains *fecB* on a BglII/Eco47III fragment from pSUSKI (A. Sauter, Microbiology/Membrane Physiology, University of Tübingen). For mutagenesis of *fecC* and *fecD*, plasmid pAS3 was used; the plasmid contains a BamHI/HindIII fragment from pT7-7 (A. Sauter). The wild-type *fecB*, *fecC*, or *fecD* gene was replaced with the mutated gene by digesting pFO76S and the plasmid carrying the mutated gene with StuI/SalI, SalI/SacI and SalI/SacI/EcoRV, respectively, and religating the fragments. The mutation sites of all constructs were confirmed by sequencing. The resulting derivatives were used to measure iron transport.

A

BtuF	70	NL**E**RIVALKPDLVIA	200	SR**E**QLVLA
FecB	91	SL**E**AIAALKPDLVIA	220	GL**E**QLAAV
FhuD	88	NL**E**LLTEMKPSFMVW	223	SI**D**RLAAY

B

BtuC	54	QI**R**LP**R**TLAVLLVGAAL	293	VA**R**LALAAA
FecC	58	NL**R**LP**R**SLVAVLIGASL	300	LA**R**ALAVPG
FecD	49	EY**R**LP**R**LLLALFVGAAL	286	LA**R**IIHPPL
FhuB(N)	56	YSLLP**R**LAISLLVGAGL	298	LT**R**VWMEVST
FhuB(C)	388	PW**R**WP**R**IMAALFAGVML	627	CG**R**MVLFPFQ

FIG. 2. Sequence alignment of the proposed interacting regions (2) of the periplasmic binding proteins BtuF, FecB, and FhuD (A) and the transmembrane proteins BtuC, FecCD, and FhuB (B) of *E. coli*. Two BtuC molecules form the transmembrane channel. FhuB is twice the size of the other transmembrane proteins, and its two halves are homologous. The amino acid residues predicted to form salt bridges between the periplasmic binding proteins and the transmembrane proteins are shown in boldface. The numbers indicate the positions in the mature proteins.

Citrate-mediated $^{55}Fe^{3+}$ transport assay. Transport of several mutant strains and the parent strain was determined in parallel. Cells of *E. coli* AA93 (Δ*fec*) (17) were transformed with the pFO76S derivatives carrying the *fecIRABCDE* genes, of which either *fecB*, *fecC*, or *fecD* was mutated. Cells were grown in 3 ml of TY medium supplemented with 1 mM sodium citrate (pH 6.8), 0.5% glucose, and 0.1 mM desferal. Citrate was added to induce the Fe^{3+}-citrate transport system, glucose was added to obtain optimal growth conditions, and desferal was added to reduce the available iron for induction. Media was inoculated to an optical density at 578 nm of 0.05, and cultures were incubated for 2 h at 37°C with shaking. Cells in the exponential growth phase were harvested by centrifugation and suspended in 3 ml of transport medium (0.1 M HEPES, 1 μg of thiamine/ml, 0.23 mM $MgCl_2$, 0.15 mM $CaCl_2$ [pH 7.2]) to an optical density at 578 nm of 0.5 to 0.6. Nitrilotriacetic acid (9 μl, 10 mM [pH 6.8]) was added to 0.9 ml of cell suspension to inhibit residual iron uptake in the absence of citrate, and the mixture was shaken for 5 min at 37°C. Transport was started by adding radiolabeled iron citrate (9 μl, 20 μM $^{55}Fe^{3+}$ hydrochloride in 1 M sodium citrate [pH 6.8]). Aliquots of 0.2 ml were withdrawn after 0, 12, 25, and 35 min and added to 5 ml of 0.1 mM LiCl in a filter device. The mixtures were filtered through nitrocellulose membranes (45-μm pore size). The filters were washed with 5 ml of 0.1 mM LiCl and then dried for 10 min at 60°C. Radioactivity on the filter was measured in a liquid scintillation counter. Transport rates were calculated by subtracting the 1-min value from the 37-min value.

Cross-linking of FecB with FecC and FecD. Cross-linking was essentially performed as described previously for cross-linking the TonB box of FecA with region 160 of TonB (18). *E. coli* BL21 *omp8* (19) was transformed with plasmids carrying pairwise combinations of the mutated *fecB* gene (one or two E-to-C replacements in FecB) with the mutated *fecC* or *fecD* gene (R-to-C replacement in FecC or FecD) cloned on plasmid pT7-7 downstream of the T7 gene 10 promoter. In the presence of rifamycin, specifically the *fec* genes are transcribed by the rifamycin-resistant phage T7 RNA polymerase; the *E. coli* RNA polymerase, in contrast, is inhibited by rifamycin. Cells were grown in 2 ml of TY medium with or without addition of 10 μM $FeCl_3$ and 1 mM citrate. Cultures in the exponential growth phase were harvested by centrifugation and suspended in 1 ml of M9 salt medium supplemented with 0.4% glucose, 0.01% methionine assay medium (Difco Laboratories), 0.01% thiamine, and 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) to induce T7 RNA polymerase synthesis with or with-

out ferric citrate supplementation. After shaking cells for 1 h at 37°C, rifamycin (10 μl of a 2-mg/ml solution in methanol) was added to allow transcription of only the *fec* genes, and incubation continued for 30 min. Prior to [^{35}S]methionine labeling of the Fec proteins, some samples were oxidized to enhance disulfide formation by incubation with 50 μM $CuSO_4$ for 5 min. [^{35}S]methionine was added to all samples, and the cultures were shaken for 10 min. Cells were collected by centrifugation and washed with 40 μl of 0.2 M Tris-HCl (pH 7.8), 0.2 mM $MgCl_2$, 20 mM NaCl, 50% glycerol, and 0.5 U of Benzonase. Cells were suspended in 40 μl of 0.2 M Tris-HCl (pH 7.8)–0.5 U of Benzonase and mixed with 40 μl of sample buffer with or without β-mercaptoethanol. Samples were heated for 3 min at 95°C, and the proteins were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by autoradiography.

RESULTS

Citrate-mediated iron transport rates of FecB, FecC, and FecD mutants. Based on the crystal structures of BtuF and BtuCD, it was proposed that BtuF and BtuC are connected by salt bridges between two glutamate residues in BtuF and several arginine residues in the BtuC dimer (Fig. 1 and 2), but it is not clear to which of the three arginine residues of BtuC the glutamate residues of BtuF bind (2). These two glutamate residues in BtuF—E72 and E202—are equivalent to the FecB glutamate residues E93 and E222, and the arginine residues in BtuC—R56, R59, and R295—are equivalent to the FecC residues R60, R63, and R302 and the FecD residues R51, R54, and R288.

To elucidate whether the equivalent residues in FecB are involved in salt bridge formation with FecC and FecD and to elucidate which of the equivalent arginine residues in FecC and FecD are involved, we introduced mutations in these residues and analyzed their effect on citrate-mediated iron transport. We constructed mutants in which E93 and E222 in FecB were replaced with alanine, cysteine, or arginine. The introduced alanine and cysteine residues in FecB cannot form salt bridges with the arginine residues in FecC and FecD, and the introduced arginine residues in FecB are expected to repulse the arginine residues in FecC and FecD. Both of these situations should reduce transport. We also constructed mutants in which one of the three arginine residues in FecC or FecD were replaced with glutamate or cysteine. If the arginine residue is involved in salt bridge formation, then glutamate introduced at this position in FecC or FecD would probably repel the glutamate in FecB, and cysteine introduced at this position in FecC or FecD should form a disulfide bridge with the cysteine introduced in place of glutamate in FecB.

Transport was determined with *E. coli* AA93 in which the entire *fec* gene cluster has been deleted. *E. coli* AA93 was transformed with plasmid pFO76S, which carries the wild-type *fecIRABCDE* genes, or with pFO76S derivatives in which the wild-type *fecB*, *fecC*, or *fecD* gene was replaced by a mutant *fecB*, *fecC*, or *fecD* gene or by combinations of mutant *fecB* and mutant *fecC* or *fecD* genes. The *fec* genes were cloned on a low-copy-number vector to approximate the chromosomal conditions. Transcription of the *fec* genes was induced with 1 mM citrate, which complexes iron in the medium. The available iron was further decreased with desferal to avoid repression of *fecIR* and *fecABCDE* gene transcription by the iron-loaded Fur repressor (1). The counts of $^{55}Fe^{3+}$ taken up by the mutants were compared to the counts taken up by the wild type determined in each experiment in parallel. A representative example of the measured iron transport is shown in Fig. 3, and all of the transport values measured are listed in Table 1.

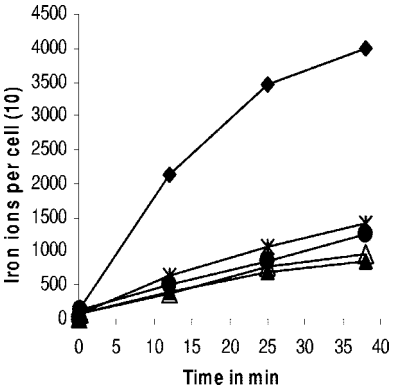

FIG. 3. Citrate-mediated $^{55}Fe^{3+}$ transport into cells of *E. coli* AA93 transformed with plasmids encoding the wild-type FecIRABCDE proteins (◆) and encoding these proteins with the following mutations: FecC(R60C) (●), FecB(E93C) FecC(R60C) (△), FecB(E222C) FecC (R60C) (∗), and FecB(E93C E222C) FecC(R60C) (▲).

Replacement of E93 or E222 in FecB with either alanine, cysteine, or arginine greatly reduced the iron transport rates. If one considers that the residual iron uptake in Δ*fecBCD* mutants amounts to ca. 5% of the uptake of wild-type *fec* and this value was not subtracted from the values listed in Table 1, the FecB(E93) mutants showed a transport of 5 to 12% and the FecB(E222) mutants showed a transport of 11 to 21% of the wild type. Of the E222 mutations, the transport was most strongly reduced in the FecB(E222R) mutant, in which the introduced arginine residue in FecB was expected to repulse the arginine residue in either FecC or FecD. The introduction of mutations in both E93 and E222 did not further reduce the transport.

The cysteine and glutamate replacements at all three arginine positions in FecC reduced the transport to about one-third of the wild-type transport, except for R63C, which had a relatively high transport of 74% compared to the wild type. Similar results were obtained with the FecD mutations. The cysteine and glutamate replacements at all three arginine positions of FecD strongly reduced iron transport, except for R288C, which had a relatively high transport of 65% compared to the wild type.

In double or triple mutants, in which one or both glutamate residues of FecB were replaced with cysteine and the arginine residue of FecC or FecD was replaced with cysteine, the iron transport was reduced to the same level as with the single mutations (Table 1).

Other mutations were introduced in FecB along the predicted interface between FecB and FecCD. Of the nine mutations examined, only two caused a strong decrease in iron transport, namely, R183C to 13% and W246C to 8% of the wild-type level (Table 1).

Cross-linking of FecB to FecCD via disulfide bond formation. FecB mutant proteins, in which one or both glutamate residues were replaced with cysteine, combined with FecC or FecD mutant proteins, in which one arginine was replaced with cysteine, were synthesized in *E. coli* BL21 and labeled with [^{35}S]methionine. The radiolabeled proteins were separated by

TABLE 1. Citrate-mediated $^{55}Fe^{3+}$ transport of FecB, FecC, and FecD mutants

Strain[a]	Transport (% of wild type)[b]
FecB(E93A)	10
FecB(E93C)	17
FecB(E93R)	13
FecB(E222A)	26
FecB(E222C)	32
FecB(E222R)	16
FecB(E93A, E222A)	14
FecB(E93C, E222C)	16
FecB(E93R, E222R)	10
FecC(R60C)	33
FecC(R60E)	29
FecC(R63C)	74
FecC(R63E)	30
FecC(R302C)	24
FecC(R302E)	28
FecD(R51C)	32
FecD(R51E)	14
FecD(R54C)	19
FecD(R54E)	24
FecD(R288C)	65
FecD(R288E)	35
FecB(E93C) FecC(R60C)	15
FecB(E222C) FecC(R60C)	24
FecB(E93C) FecC(R63C)	20
FecB(E93C, E222C) FecC(R60C)	21
FecB(E93C) FecC(R302C)	14
FecB(E222C) FecC(R302C)	21
FecB(E93C, E222C) FecC(R302C)	21
FecB(E93C) FecD(R51C)	26
FecB(E222C) FecD(R51C)	21
FecB(E093C, E222C) FecD(R51C)	26
FecB(E93C) FecD(R54C)	19
FecB(E93C) FecD(R288C)	31
FecB(E222C) FecD(R288C)	28
FecB(E93C, E222C) FecD(R288C)	26
FecB(R66C)	61
FecB(K68C)	100
FecB(R69C)	100
FecB(E93R	77
FecB(Q87C)	88
FecB(R183C)	13
FecB(R245C)	95
FecB(W246C)	8
FecB(Q248C)	92

[a] The form "FecB(E93A)" indicates that E93 was replaced by A, etc.

[b] Transport is given as the 37-min radioactive counts taken up by the cells from which the 1-min counts were subtracted. Values are the average of three to six determinations. The uptake into strains lacking FecB, FecC, or FecD was ca. 5% of the wild-type rate and was not subtracted from the given values.

sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the absence of β-mercaptoethanol to examine whether proteins complexes of approximately 65 kDa were formed, i.e., FecBC and FecBD (molecular masses: FecB, 31 kDa; FecC, 35 kDa; and FecD, 34 kDa). Copper sulfate was added to enhance disulfide formation. The FecA protein was present in all samples and served as a standard for the amount of protein applied to the gels. The wild-type proteins did not form disulfide bridges, whereas samples containing FecB(E222C) and FecC (R60C) and samples containing FecB(E93C) and FecD(R54C) contained increased amounts of a protein of approximately 65 kDa (data not shown). This result suggested that FecB docks on FecCD such that E222 of FecB comes in contact with FecC(R60) and E93 comes into contact with FecD(R54). This interaction was not facilitated by the addition of the substrate ferric citrate to the medium). The same cross-linked bands were seen when no copper sulfate was added, but the samples with ferric citrate were weaker than those without ferric citrate (not shown). The yields of the cross-linked band were low, which is usually observed in in vivo cross-linking experiments and may also be caused by a temporary interaction of FecB with FecCD.

DISCUSSION

Our analyses revealed that the amino acid residues predicted to be involved in the interaction between the periplasmic binding protein FecB and the transmembrane proteins FecCD do actually contribute to the docking of FecB to FecC and FecD.

The glutamate residues E93 and E222 of FecB are involved in the interaction based on the strong reduction of Fe^{3+}-citrate transport of mutants with amino acid replacements in these residues. Mutation of both glutamate residues in FecB reduced the transport (10 to 16% of the wild-type level) to the same level as the lowest transport of the single mutations (10 to 17% of the wild-type level), i.e., the effects of the single mutations were not additive in the double mutants. We therefore consider that such low transport levels, between 10 and 20% of the wild-type level, indicate docking of FecB to FecCD without the involvement of the salt bridges formed between the glutamate and arginine residues in the wild-type proteins.

The arginine residues R60 and R302 in FecC and R51 and R54 in FecD are also involved based on the reduction of transport levels of mutants in these residues (ca. 30% of the wild-type level). Transport of the FecC(R63E) and the FecD(R288E) mutants also was reduced to 30% of the wild-type level, probably owing to repulsion of the glutamate residue in FecB, which indicates that R63 in FecC and R288 in FecD are also involved in the interaction. However, because transport of the FecC(R63C) and FecD(R288C) mutants was only slightly reduced (74 and 65%, respectively, of the wild-type level), the contribution of these residues is probably lower than that of the other two arginine residues in FecC and in FecD, which were strongly affected by both glutamate and cysteine replacements.

The reduction in transport activity to ca. 30% of the wild-type level of the FecC and FecD mutants was less pronounced than the reduction to 10 to 17% of the wild-type level of the FecB mutants. The single arginine replacements in FecC or FecD might have less effect because the remaining two arginine residues in FecC or FecD at the predicted docking site still contribute to the attraction of the glutamate residues of FecB.

R183 and W246 of FecB are most likely also involved in the interaction, as suggested by low levels of transport of the FecB(R183C) and FecB(W246C) mutants (13 and 8%, respectively, of the wild-type level). These sites are predicted to be close to the interface between FecB and FecCD, based on the possibly analogous BtuFC structures. However, these mutations might not indicate sites of interaction of FecB and FecCD but might rather alter the conformation of FecB so that binding of FecB to FecCD is impaired.

To determine which glutamate residue of FecB interacts

with FecC and FecD, respectively, we analyzed mutants in which the amino acid residues in all three proteins predicted to be involved in the salt bridge formation were replaced with cysteine residues. If these positions in the proteins are close enough to interact with each other as predicted, disulfide bonds between the introduced cysteine residues should form. Such spontaneous in vivo cross-linking was reproducibly observed between FecB(E93C) and FecD(R54C) and between FecB(E222C) and FecC(R60C). These results suggest that in the wild-type proteins FecB(E93) forms a salt bridge with FecD(R54) and FecB(E222) forms a salt bridge with FecC(R60).

Disulfide bond formation was not enhanced by ferric citrate, which one would expect if FecB with bound ferric citrate preferentially binds to FecCD. However, when copper sulfate was added to increase oxidation of the cysteine residues to disulfides, disulfide bond formation was enhanced by ferric citrate. Binding of FecB without bound ferric citrate to FecCD was not unexpected because the structure of siderophore-binding proteins differs from that of most other binding proteins. For example, isolated binding protein FhuD placed in the periplasm of spheroplasts prevents the cytoplasmic membrane transporter protein FhuB from being degraded by added trypsin or proteinase K, regardless of whether FhuD is loaded with its substrate aerobactin; and FhuD chemically cross-links to FhuB to a higher degree in the absence of aerobactin than in its presence (21). This finding that substrate-loaded and unloaded FhuD binds its cognate transporter FhuB in the periplasm is supported by crystal structures and molecular dynamic simulations of substrate-loaded and unloaded FhuD (8, 14). FhuD assumes a rigid structure that differs in the substrate-loaded and unloaded forms much less than is observed for most other binding proteins. FhuD resembles BtuF and other binding proteins of organic metal ion complexes, classified as cluster 8 binding proteins. FecB also belongs to this cluster, which indicates that it would bind to FecCD regardless of whether it is loaded with its substrate ferric citrate or not. However, substrate loading of FecB must also be recognized by FecCD since only substrate-loaded binding proteins initiate the ATP hydrolysis necessary for transport across the cytoplasmic membrane (9).

The results described here identify the amino acids that position a binding protein to its transmembrane transport proteins. The results probably apply to all cluster 8 binding proteins (23). For example, amino acid replacements of the sites E97 and E231 in the FhuD2 binding protein of the *Staphylococcus aureus* ferric hydroxamate transport system, guided by the crystal structures of the vitamin B_{12} transporters, resulted in mutants that showed a reduced growth promotion by ferric hydroxamates in a plate bioassay (22). E97 and E231 are equivalent to FecB(E93) and FecB(E222) and thus belong to the conserved glutamate residues of cluster 8 binding proteins implicated in interaction with the transmembrane proteins. FhuD2 could be modeled according to the known crystal structure of the *E. coli* FhuD (22). Previously, genetic suppression analysis was used to identify possible sites of interaction between binding proteins and transmembrane proteins. For example, mutated histidine binding protein HisJ(R176S) did not support histidine uptake into *Salmonella enterica* serovar Typhimurium. Mutant HisP(T205A) in one of the two transmembrane proteins restored histidine uptake in an allele-specific way (20). In the maltose transport system of *E. coli* inactive *malF* and *malG* mutants encoding the MalF G transmembrane proteins were allele specifically suppressed by *malE* mutations encoding the MalE binding protein (24). However, in contrast to FhuD and FecB, HisJ and MalE belong to the cluster 1 of binding proteins that change considerably, like a Venus fly trap, the structure upon binding of their substrates. FhuD and most probably also FecB contains a rigid α-helix that connects the two lobes that move in the class 1 binding proteins and considerably restricts their movement (8). Therefore, interaction between the cluster 1 and cluster 8 binding proteins with their transmembrane proteins certainly differs. The structural diversity of ABC transporters was recently exemplified by the crystal structure of the putative molybdate transporter $ModB_2C_2$ of *Archaeoglobus fulgidus* in complex with its binding protein ModA, which strongly differs from the known crystal structures of ABC transporters (13).

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